

Cloning and Characterization of the Amino Terminal 30% of Retinoid and Fatty Acid

Binding Glycoprotein in Schneider's S2 Cells

**Honors Project**

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## ABSTRACT

### CLONING AND CHARACTERIZATION OF THE AMINO TERMINAL 30% OF RETINOID AND FATTY ACID BINDING GLYCOPROTEIN IN SCHNEIDER'S S2 CELLS

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MAY 02, 2009

Our lab is interested in exploring insect lipid transporting genes and the lipoproteins they produce within *Drosophila melanogaster*. We want to study lipid transporting genes in insects because several labs have demonstrated that components necessary for the synthesis and secretion of insect lipoproteins are similar to those required for production of low-density lipoproteins (LDL) in humans. This is of particular importance given that elevated LDL concentration in human plasma is known to lead to atherosclerosis and heart disease. It is our lab's intent to develop models for the exploration of lipoprotein biogenesis using *D. melanogaster* in expectation that our findings may contribute to further understanding of human LDL production. At present, our interest centers on the production of insect apolipoprotein II/I, named retinoid and fatty acid binding glycoprotein (RFABG) in *D. melanogaster*. RFABG is a homolog of the major structural protein on human LDLs, apolipoprotein B, and is known to transport fatty acids to insect flight muscle during extended flights. As a result we have chosen to subclone an N-terminal portion of RFABG, which constitutes about 30% of the entire DNA sequence (RF30). Through sequential digests and purification procedures, our lab successfully transferred the entire RF30 sequence into the insect expression vector, pRmHa-3. We have also appended a short, engineered sequence to RF30 that encodes the FLAG epitope tag and a termination codon. Future efforts may now explore expression of this FLAG-tagged RF30 construct in Schneider's S2 cells to determine whether these cells are capable of producing lipoproteins.

## INTRODUCTION

Atherosclerosis is the process by which fatty deposits, calcium, cellular waste products, and cholesterol accumulate along the inner walls of an artery. Over time these deposits, collectively called plaque, can considerably disrupt the natural flow of blood through the artery. This inhibited blood flow facilitates the formation of blood clots that could potentially relocate to other parts of the body, in turn hindering more blood flow. If such a clot were displaced to an artery supplying as vital an organ as the heart or brain, massive cardiac arrest or stroke could result. In addition, an over accumulation of plaque could render the arteries fragile, making them more susceptible to ruptures or cracks. Although other factors exist, a major contributor to the development of atherosclerotic lesions is an elevated plasma concentration of low-density lipoprotein (LDL) associated cholesterol.

The major function of lipoproteins is to manage the transportation of fats, cholesterol, and other metabolic wastes throughout the circulatory system. Our lab is interested in exploring insect lipid transporting genes and the lipoproteins they produce within *Drosophila melanogaster*, which serves as a model organism with certain analogous features to humans. We want to study insect lipid transporting genes and the proteins produced by these insects because several labs have demonstrated that components necessary for the synthesis and secretion of insect lipoproteins are very similar to those necessary for production of LDLs in humans (1). It is our lab's intent to develop models for the exploration of lipoprotein biogenesis using *Drosophila melanogaster* in expectation that our findings may contribute to our further understanding

of human LDL secretion. As of now, our interest centers around the production of apolipoprotein II/I (apoLp-II/I), specifically named Retinoid Fatty Acid Glycoprotein (RFABG) for *Drosophila melanogaster*. RFABG is a homolog of the major structural protein on human LDLs, apolipoprotein B (apoB) (2). RFABG contributes to the formation of high density lipoprotein (HDLp) in insects. The HDLp is then filled with additional diglycerides by a lipid transfer particle within the insect fat body to become low density lipoprotein (LDLp). This process is essential for insects as they rely on the production of LDLp as the source of energy for flight muscle.

Our lab has successfully transferred a cDNA sequence of RFABG into an insect expression vector to further investigate insect lipoprotein biogenesis and secretion. Since all large lipid transporting proteins, within both vertebrates and invertebrates, share similarities concentrated in their N-terminal region (5), we are using the N-terminal portion of RFABG, which is about 30% of the entire DNA sequence (RF30) originally cloned in a mammalian expression vector, pCMV5. Through sequential digests and purification procedures, we have transferred the approximately 3,000 base pair RF30 sequence to an insect expression vector, pRmHa-3. This expression vector was constructed to include an ampicillin resistance gene to serve as a selectable marker for detection and a metallothionein promoter to allow for later induction of expression of a gene under control of the promoter through metal ion inductions (3). Throughout the cloning process we chose to use restriction endonucleases that were unique to pRmHa-3 but also would excise the intact RF30 sequence from the mammalian expression vector. We have also appended a short, engineered sequence to RF30 that encodes the eight amino acid FLAG epitope tag and an engineered termination codon. We now wish to

explore the expression of this FLAG-tagged RF30 construct in Schneider's S2 cells to determine whether these cells are capable of producing lipoproteins.



## **MATERIALS AND METHODS**

### **RF30 Excision From pCMV-5**

An ApaI (50 units/ $\mu$ l) digest of 2 $\mu$ g of pCMV-5 containing RF30 (1 $\mu$ g/ $\mu$ l) was performed at an adjusted reaction volume of 20 $\mu$ l according to New England Biolab's (NEB) protocol for 1 hour at 37°C. The reaction was adjusted to 1X TBE and 50  $\mu$ l was divided equally in two wells (~1 $\mu$ g DNA per well) of a 0.7% agarose gel. The gel was electrophoresed at 140 volts for 45 minutes and analyzed by staining with SYBR-Safe (Invitrogen). The digested fragment was excised and then purified with Wizard® SV Gel Clean-Up System (Promega) and the DNA was recovered via centrifugation protocol with a final eluted volume of 12 $\mu$ l (~2 $\mu$ g). The ApaI digested pCMV-5 containing RF30 was then blunt ended with NEB Quick Blunting Kit in a 25 $\mu$ l reaction which was electrophoresed and then purified via centrifugation procedure of the Wizard® SV Gel Clean-Up System. A BamHI (20 units/ $\mu$ l) digestion was performed with the eluted DNA in a 40 $\mu$ l reaction overnight at 37°C. This final digestion released RF30 from the plasmid and the ~3,000bp fragment was excised from a gel, purified, DNA eluted, and then retained at -20°C until needed for a ligation procedure.

### **Insect Expression Vector Preparation**

An EcoRI (20units/ $\mu$ l) digest of 2 $\mu$ g of pRmHa-3 (1 $\mu$ g/ $\mu$ l) was performed at an adjusted reaction volume of 20 $\mu$ l according to New England Biolab's (NEB) protocol for 1 hour at 37°C. The reaction was adjusted to 1X TBE and 50  $\mu$ l was divided equally in two wells (~1 $\mu$ g DNA per well) of a 0.7% agarose gel. The gel was electrophoresed at 140 volts for

45 minutes and analyzed by staining with SYBR-Safe (Invitrogen). The digested fragment was excised and then purified with the Wizard® SV Gel Clean-Up System (Promega) and the DNA was recovered via centrifugation protocol with a final volume of 12µl (~2µg) eluted. The EcoRI digested pRmHa-3 was then blunt ended with NEB Quick Blunting Kit in a 25µl reaction which was electrophoresed and then purified via centrifugation procedure of the Wizard® SV Gel Clean-Up System. A BamHI (20 units/µl) digestion was performed with the eluted DNA in a 40µl reaction overnight at 37°C. This final digestion was excised from a gel, purified, eluted and then retained at -20°C until needed for the ligation procedure.

### **Ligation, Transformation and plasmid DNA Mini-preparation**

Approximately 50ng of the prepared insect expression vector, pRmHa-3, and ~120ng of the RF30 insert were combined for NEB's Quick Ligation™ Kit and the procedure for ligation of the two DNA fragments were followed as provided by the manufacturer for 15 minutes at room temperature. Chemically competent XL1-Blue *E. coli* were transformed with the product of the ligation procedure. Transformed cells were selected by growth on Luria-Bertani agar plates containing 50µg/mL ampicillin. Single colonies were selected and grown to stationary phase for plasmid DNA mini-preparations according to the alkaline lysis protocol (4).

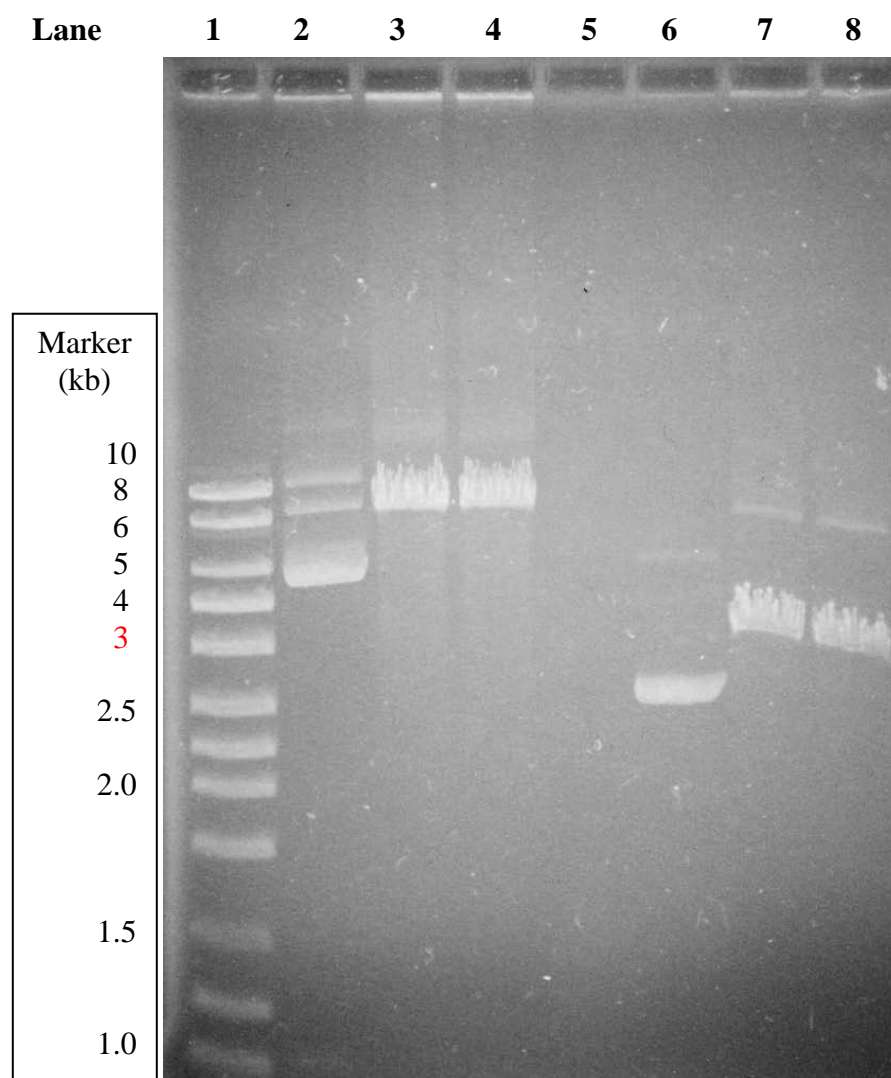
### **FLAG Epitope Tag**

An engineered sense strand, oligo 5' GA TCC GAC TAC AAG GAT GAC GAT GAC AAG TAA G 3' (5µg), and antisense strand, oligo 5' TC GAC TTA CTT GTC ATC GTC ATC CTT GTA GTC G 3' (5µg), were hybridized in 0.5 M TRIS (pH 7.5) in a final

reaction volume of 110 $\mu$ l at 90°C for 10 minutes after which the samples were allowed to come to room temperature. After hybridization, the double stranded oligo was purified using the Wizard® SV Gel Clean-Up System and adjusted to 1 $\mu$ g/ $\mu$ l. Approximately 2 $\mu$ g of the RF30 clone was prepared for ligation with the oligo by a double digestion with SalI (20units/ $\mu$ l) and BamHI (20units/ $\mu$ l) at an adjusted reaction volume of 20 $\mu$ l according to New England Biolab's (NEB) protocol for 1 hour at 37°C. Approximately 50ng of the SalI and BamHI digested RF30 clone, and ~40ng of the double stranded oligonucleotide were combined for NEB's Quick Ligation™ Kit and the procedure for ligation of the two DNA fragments were followed as provided by the manufacturer for 15 minutes at room temperature.

## RESULTS

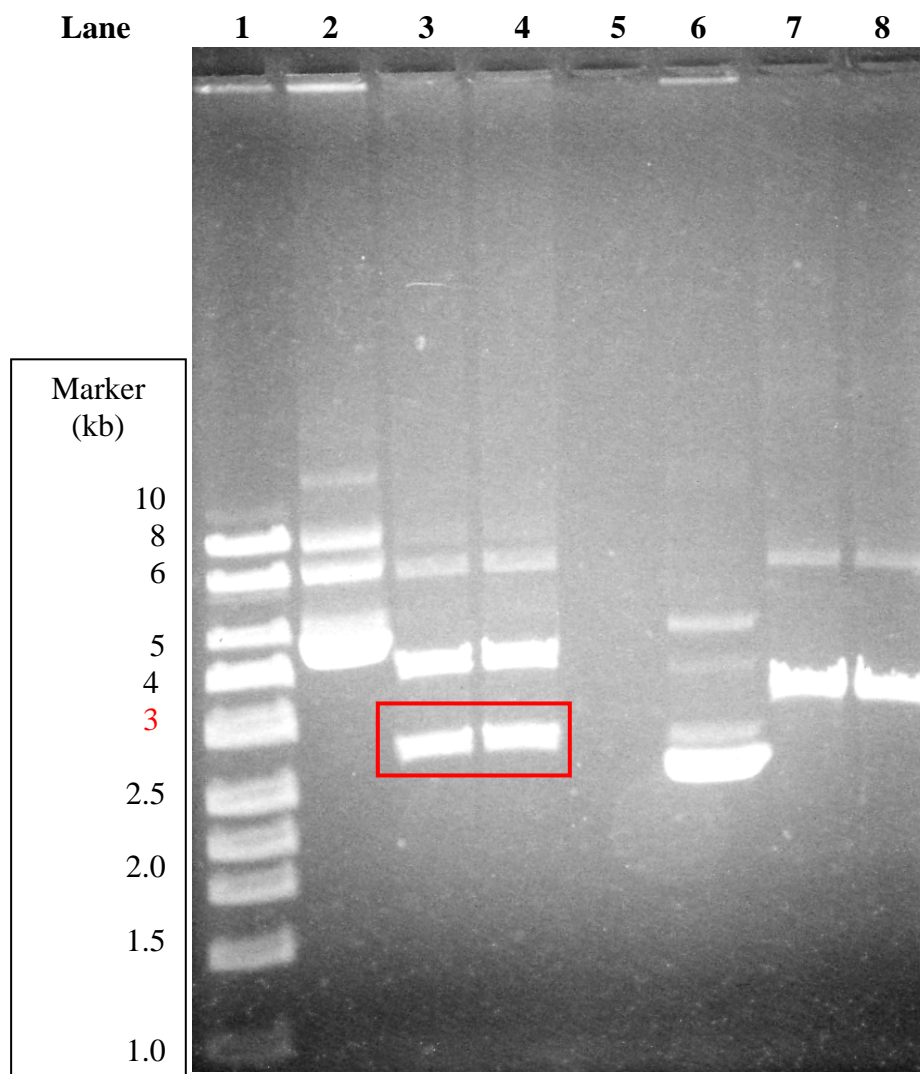
**Figure 1**



**Figure 1.** Agarose gel + SYBR-Safe stain. Lane 1 is 1 kb DNA marker. Lane 2 is undigested pCMV5 containing RF30. Lanes 3-4 contain pCMV5 digested with *Apa*I.

Lane 5 is empty. Lane 6 is undigested pRmHa-3. Lanes 7-8 contain pRmHa-3 digested with EcoRI.

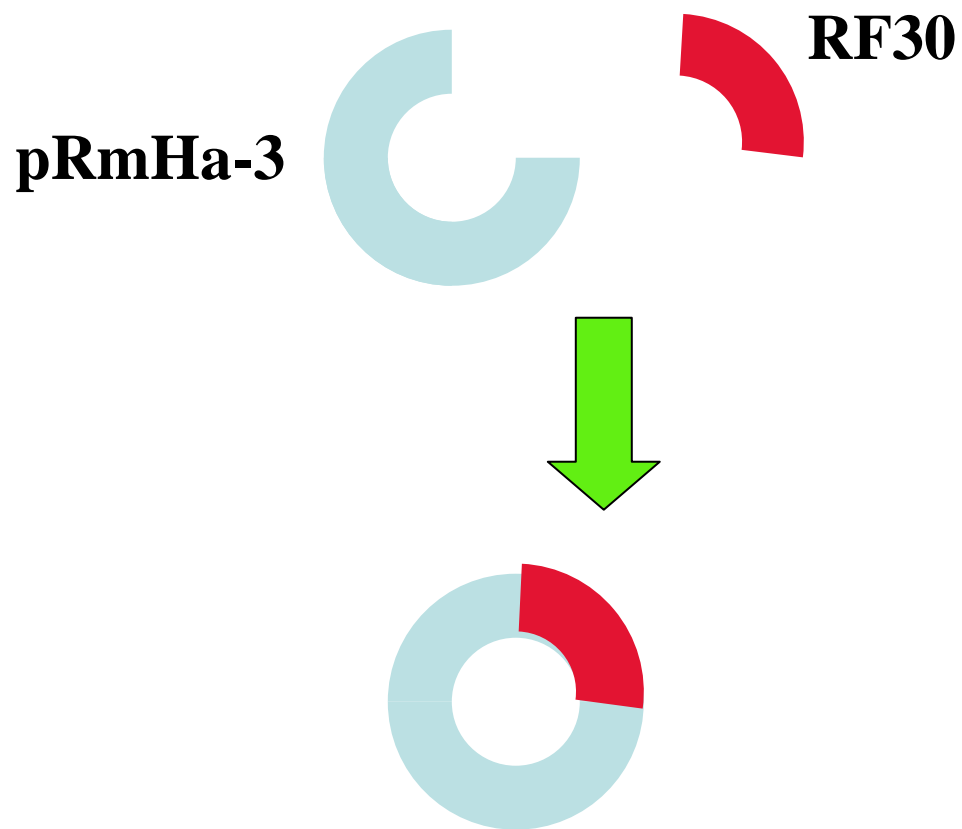
**Figure 2**



**Figure 2.** Agarose gel + SYBR-Safe stain. Lane 1 is 1 kb DNA marker. Lane 2 is undigested pCMV5 containing RF30. Lanes 3-4 contain pCMV5 digested with BamHI.

Lane 5 is blank. Lane 6 is undigested pRmHa-3. Lanes 7-8 contain pRmHa-3 digested with BamHI.

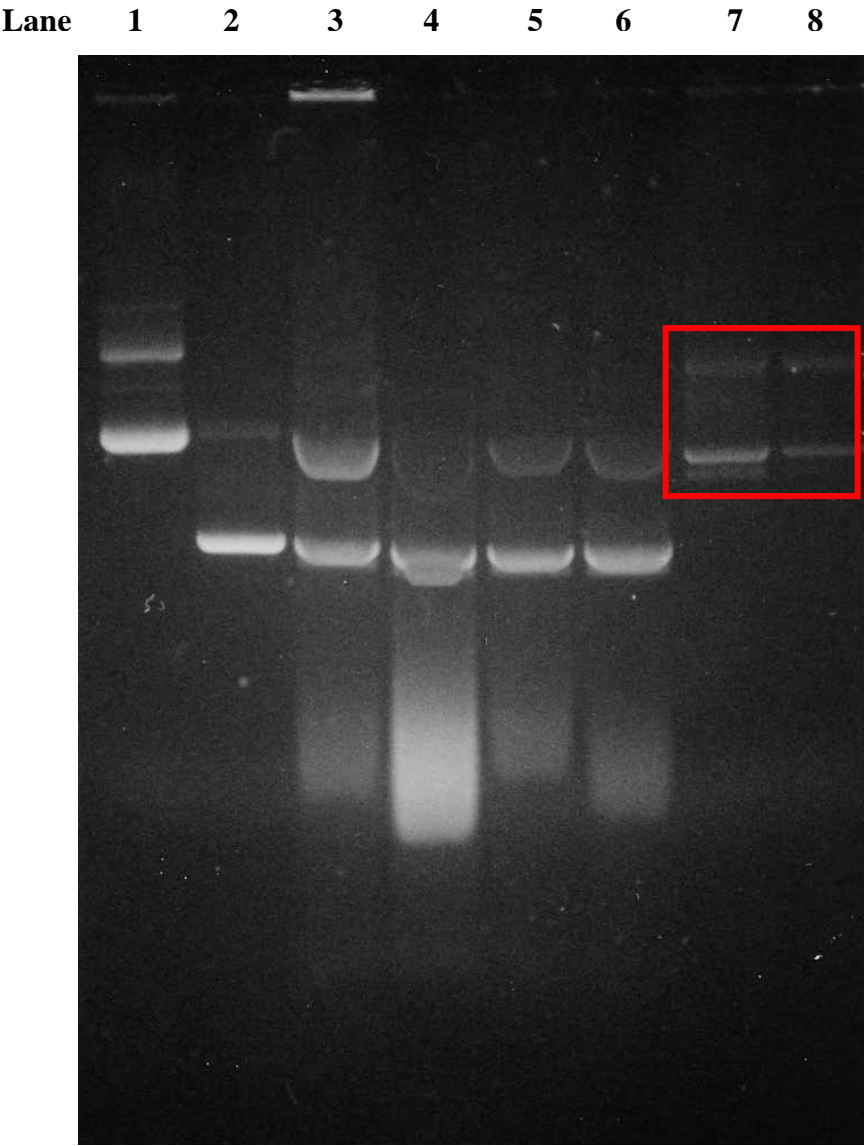
**Figure 3**



**Figure 3. Ligation of Vector and Insert.**

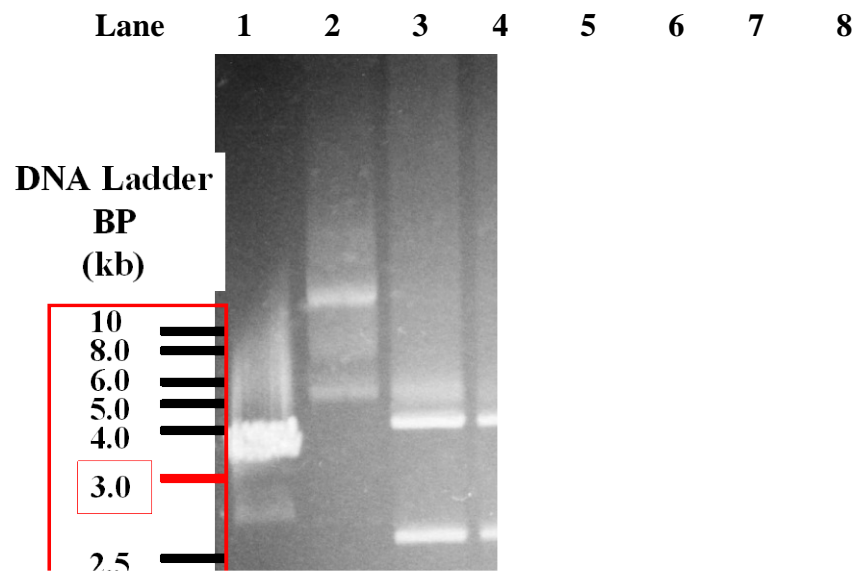
Visualization of the ligation of prepared pRmHa-3 and RF30 DNA sequence.

**Figure 4**



**Figure 4. Plasmid DNA Minipreparations** Agarose gel + SYBR-Safe stain. Lane 1 undigested pCMV5 containing RF30. Lane 2 is undigested pRmHa-3. Lanes 3-8 are DNA mini-preparations 1-6, respectively

**Figure 5**



**Figure 5. Analytical Digestion** Agarose gel + SYBR-Safe stain. Lane 1 BamHI digested pRmHa-3. Lane 2 is undigested pRmHa-3 containing RF30. Lanes 3-4 BamHI and EcoRV digested clone. Lane 5 NEB's 1kb DNA Ladder. Lane 6 PacI digested clone. Lane 7 NcoI digested clone. Lane 8 pCMV5 containing RF30 NcoI digest.



## CONCLUSION/DISCUSSION

The analytical digest (Figure 4) validates that our lab has produced a pRmHa-3 containing RF30F clone. Several different restriction enzymes were used to conclude that the RF30 insert was not within the pCMV5 vector. Lanes 3-4 each depict two distinct bands, one of which is ~4,600 base pairs, the other band is ~2,300 base pairs. The presence of these bands after double digestion, verify that the clone contains an insert of the correct size and that the RF30 insert is present. To further validate our clone, PacI was used in Lane 6 to determine which vector held the insert. Both plasmid maps indicate that this restriction enzyme will not cut pCMV5 and only cuts pRmHa-3 clone once, as Figure 4 indicates. The plasmid maps also revealed that NcoI does not digest pRmHa-3, as indicated by Figure 4, Lane 7, but does hold three restriction sites in pCMV5.

Now that we have obtained our clone and FLAG epitope tagged RF30, we will use it to transfect Schneider's S2 cells through a suggested protocol (3). We will acquire expression of our RF30 DNA sequence through the metallothionein promoter within pRmHa-3 by induction with copper sulfate. We will then collect data on the protein secretion from the cells via antibody detection techniques with the appended FLAG epitope tag. This will further characterize the role of insect lipid transporting genes for a more native analysis of their biogenesis.

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